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## Hormonal regulation of leptin receptor expression in primary cultures of porcine hepatocytes<sup>☆</sup>

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### Abstract

A study was conducted to elucidate hormonal control of leptin receptor gene expression in primary cultures of porcine hepatocytes. Hepatocytes were isolated from pigs (52 kg) and seeded into collagen-coated T-25 flasks. Monolayer cultures were established in medium containing fetal bovine serum for 1 day and switched to a serum-free medium for the remainder of the 3-day culture period. To establish basal conditions hepatocytes were maintained in serum-free William's E medium containing 10 nM dexamethasone and 1 ng/ml insulin. For the final 24 h, insulin (1 or 100 ng/ml) or glucagon (100 ng/ml), were added in the presence or absence of 100 nM triiodothyronine (T3). RNA was extracted and quantitative RT-PCR was performed with primers specific for the long form and total porcine leptin receptors. Leptin receptor expression was calculated relative to co-amplified 18S rRNA. Expression of the long form of the leptin receptor was confirmed under basal conditions. Insulin, glucagon and synthetic human proteins (ghrelin and GLP-1) at 100 ng/ml had no influence on leptin receptor expression; the addition of T3 was associated with a marked increase ( $P < 0.001$ ) in expression of total and long forms of the leptin receptor by 1.6 and 2.4-fold, respectively. Addition of leptin to cells which were pre-treated with T3 for 24 h (to up-regulate leptin receptor expression), confirmed the lack of a direct effect of leptin on glucagon-induced glycogen turnover and cAMP production. These data suggest that porcine

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hepatocytes may be insensitive to leptin stimulation even when leptin receptor expression is enhanced by T3.

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## 1. Introduction

Leptin is a 16 kDa polypeptide hormone secreted primarily by adipose tissue which plays a major role in feed intake and energy metabolism [1]. While there is a great deal of information concerning the central role of leptin in brain and neural tissue, much less is known about its action in peripheral tissues. The fully active leptin receptor has been identified as a transmembrane protein in the class 1 cytokine receptor family [2,3]. In addition, other shorter isoforms which are products of alternative RNA splicing of intracellular domains, have been identified in many tissues and may retain partial signaling activity [4–6]. Long form leptin receptor expression has been identified and evaluated in many tissues from the pig including brain, muscle, fat and liver [2,7]. To assess the potential influences of leptin and other hormones on porcine hepatic energy metabolism, we have developed a hepatocyte isolation and culture system for larger pigs (50–70 kg) that is highly sensitive to polypeptide hormone input. However, in our previous studies we could not define any specific roles for leptin in hepatocyte energy metabolism [8]. This is in sharp contrast to rodent liver where leptin has been shown to both enhance and antagonize insulin action on glucose and lipid metabolism [9–14]. Recently, Raman et al. [15] demonstrated expression of the long form of the leptin receptor in porcine hepatocytes prepared from suckling pigs and maintained in vitro for 24–48 h, but were also unable to define any direct effects of leptin on glucose metabolism. The goal of this investigation was to establish if the leptin receptor gene was indeed expressed by hepatocytes in our 72 h culture system with cells prepared from larger metabolically-mature animals. In addition, we attempted to up-regulate leptin receptor expression with optimal levels of T3 in an effort to enhance the possible effects of leptin on cAMP production and glycogen metabolism.

## 2. Materials and methods

### 2.1. Hepatocyte isolation

Crossbred barrows (11 total,  $52 \pm 4$  kg;  $n$ =three to five pigs per experiment) were stunned by electric shock and exsanguinated. Livers were immediately excised and the left lateral lobe was removed. Care and treatment of all pigs was approved by the Institutional Animal Care and Use Committee of the U.S. Department of Agriculture. Hepatocytes were isolated by a two-step collagenase digestion procedure essentially as previously described [8,16] where only a small portion (approximately 85 g) of the lobe was used. Viability of hepatocytes isolated by low speed centrifugation was  $87.1 \pm 1.3\%$  by trypan blue dye exclusion.

## 2.2. Hepatocyte culture

Hepatocytes ( $4.5 \times 10^6$ ) were seeded into T-25 flasks, pre-coated with 150  $\mu$ g pig tail collagen. Cells were initially maintained at 37 °C and 5% CO<sub>2</sub> in William's E medium containing 10% fetal bovine serum (FBS) and amended with ITS (insulin:transferrin:selenium, 2.5  $\mu$ g/ml:2.5  $\mu$ g/ml:2.5 ng/ml),  $\beta$ -mercaptoethanol (100  $\mu$ M), glutamine (2 mM) and antibiotics (gentamicin, penicillin, streptomycin, and amphotericin B). Following a 3 h attachment period, flasks were washed twice with HEPES-buffered saline and William's E medium, containing 5% FBS, was added to each flask. On the following day, flasks were washed twice and media was replaced with serum-free William's E medium containing 10 nM dexamethasone, 100  $\mu$ M  $\beta$ -mercaptoethanol, 10 mM HEPES, 10 nM Na<sub>2</sub>SeO<sub>3</sub>, 1 mM carnitine, 2 mM glutamine, antibiotics, 0.01% DMSO, 0.1% BSA, and 1 ng/ml bovine insulin (basal condition). The following day, cell monolayers were washed and fresh medium containing the indicated hormone additions was added for a final 24 h period. Polypeptide hormones were used at 100 ng/ml and triiodothyronine (T3) was added at levels between  $10^{-9}$  and  $10^{-6}$  M. Except where noted, all media, cell culture and assay reagents were purchased from Sigma (St. Louis, MO). Recombinant human [rh]-leptin (Calbiochem, La Jolla, CA), h-octanoyl ghrelin (Tocris Cookson, Ltd., Bristol, UK) and h-glucagon-like peptide-1 (GLP-1, 7–37 amide; Phoenix Pharmaceuticals, Inc., Belmont, CA) were solubilized, aliquoted for single use application and stored at –80 °C. After 3 days of culture, cell monolayers were washed with ice-cold glucose-free HEPES-buffered saline. Cells from each flask were scraped in either two ml of glucose-free HEPES-saline, 0.1N HCl or TRI-Reagent (Invitrogen, Carlsbad, CA), depending on the experiment. All cell material was stored at –80 °C prior to analysis. Porcine hepatocytes maintained under basal conditions (between 3 and 72 h) are shown in Fig. 1.

## 2.3. Analytical

### 2.3.1. Protein

Protein in cell homogenates was determined by a modified Lowry procedure following NaOH solubilization of TCA-precipitated material [17]. Bovine serum albumin was used as a standard.

### 2.3.2. Glycogen

The glycogen in cell homogenates was determined by a modification of the method of Nguyen et al. [18]. Briefly, samples were incubated in the absence (background) or presence of amyloglucosidase (E.C.3.2.1.3) for 1 h at 37 °C, to liberate glucosyl units from glycogen. Glucose was subsequently assayed in microtitre plates by a modification of an enzymatic method (SIGMA Diagnostics #18-UV). Background glucose content was subtracted to obtain cellular glycogen content and data were expressed as  $\mu$ g glucosyl units released from glycogen per mg cell protein.

### 2.3.3. Adenosine 3',5'-cyclic monophosphate (cAMP)

Following the short-term incubations (20 or 90 min) with or without glucagon, culture flasks were placed on ice, washed twice with iced buffer and frozen (–80 °C) in

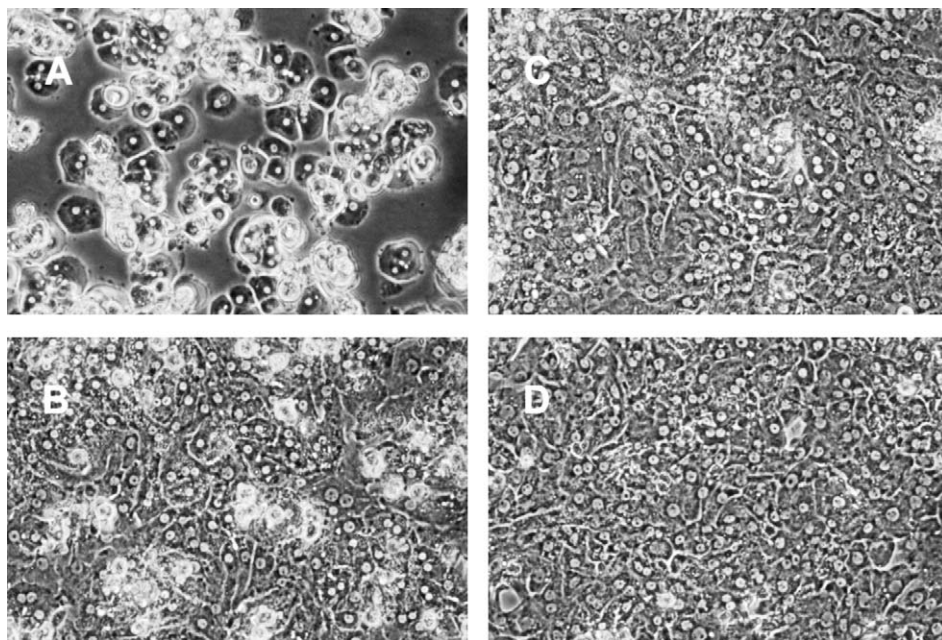


Fig. 1. Morphology of porcine hepatocyte monolayers maintained in William's E medium for 72 h on collagen. Hepatocytes were initially seeded and allowed to attach to the flask surface for 3 h in the presence 10% FBS (A). Cell debris was removed and media containing 5% FBS was added for an additional 14–16 h (B). Cell monolayers were washed and serum-free basal medium (1 ng/ml insulin) was added for an additional 24 (C) and 48 h (D).

0.1N HCl. Homogenates were prepared by sonication and the concentration of cAMP was determined by competitive ELISA (Correlate-EIA, direct cAMP, Assay Designs, Inc., Ann Arbor, MI) and standard curves were prepared and interpolated using Assay Zap v 3.1 (Biosoft, Inc., Ferguson, MO). Data are expressed as pmol cAMP/mg cell protein.

#### 2.3.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from hepatocytes using the Tri-Reagent procedure (Invitrogen) and quantification of leptin receptor expression was essentially as previously described [3]. Reverse transcription (RT) reactions (20  $\mu$ l) consisted of: 1  $\mu$ g total RNA, 50 units SuperScript II reverse transcriptase (Invitrogen), 40 units of an RNase inhibitor (Invitrogen), 0.5 mM dNTPs, and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 25  $\mu$ l containing: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.0  $\mu$ l of the RT reaction, 1.0 unit of Platinum Taq DNA polymerase (hot start), 0.2 mM dNTPs, 2.0 mM  $Mg^{2+}$  (Invitrogen), 10 pmol each of gene specific primers (total and long form specific) and 5 pmol each of a primer/competimer mixture (3:7) specific for 18S rRNA (Universal 18S kit, Ambion, Inc., Austin, TX). Thermal cycling parameters were: 1 cycle 94 °C for 2 min, followed by 35 cycles of: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final

extension at 72 °C for 8 min. The following primers were used:

*Total leptin receptors* (393 bp product):

Sense: 5'-ACTGGAGCACCCCTTTACT-3'

Antisense: 5'-TGGTTGACCATCTGCAAGTC-3'

*Long form specific* (396 bp product):

Sense: 5'-CAGTGACATTTGGCCCTCTT-3'

Antisense: 5'-AGGCCTGGGTTTCTATCTCC-3'

The identity of both PCR amplicons (long form and total) as fragments of the porcine leptin receptor gene was confirmed by subjecting each of the cDNAs produced by RT-PCR to direct bi-directional automated fluorescent DNA sequencing utilizing a Beckman Coulter CEQ 8000XL Genetic Analysis System using a dye terminator cycle sequencing kit (Beckman Coulter, Inc., Fullerton, CA).

#### 2.3.5. Capillary electrophoresis-laser induced fluorescence (CE-LIF)

Aliquots (2 µl) of RT-PCR samples were diluted 1:100 with dH<sub>2</sub>O prior to CE-LIF. A P/ACE MDQ (Beckman Coulter, Inc.) equipped with an argon ion LIF detector was used. Capillaries were 75 µm i.d. × 32 cm µSIL-DNA (J&W Scientific, Folsom, CA). Enhance dye (Beckman Coulter, Inc.) was added to the DNA separation buffer (Sigma, St. Louis, MO) to a final concentration of 0.5 µg/ml. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 5 min. P/ACE MDQ software (Beckman Coulter, Inc.) was used to calculate peak areas for the PCR products separated by CE. The level of gene expression was determined as the ratio of integrated peak area for each individual gene PCR product relative to that of the co-amplified 18S rRNA internal standard.

#### 2.4. Statistical analysis

Results are expressed as mean ± S.E.M. of duplicate or triplicate flasks from three to five independent hepatocyte culture preparations as indicated, and *n* is the number of animals (preparations) used in each experiment. Statistical analysis was performed using GLM ANOVA and the means were separated by using either The Student's planned multiple comparison test (basal condition versus each group) or the Tukey–Kramer Multiple Comparison Test (Number Cruncher Statistical Systems, Kaysville, UT).

### 3. Results

The long form of the leptin receptor was expressed in porcine hepatocyte cultures maintained for 48 h under basal serum-free conditions (Fig. 2). Incubation with insulin, glucagon, ghrelin and GLP-1 at 100 ng/ml for the final 24 h of culture had no influence on leptin receptor expression. However, addition of 100 nM triiodothyronine (T3) was associated with an overall 2.4-fold increase in long form leptin receptor expression ( $P < 0.001$ ) under basal conditions (low insulin) and with high insulin or glucagon. When the expression of all or total forms of the leptin receptor were evaluated, T3 was associated with enhanced expres-

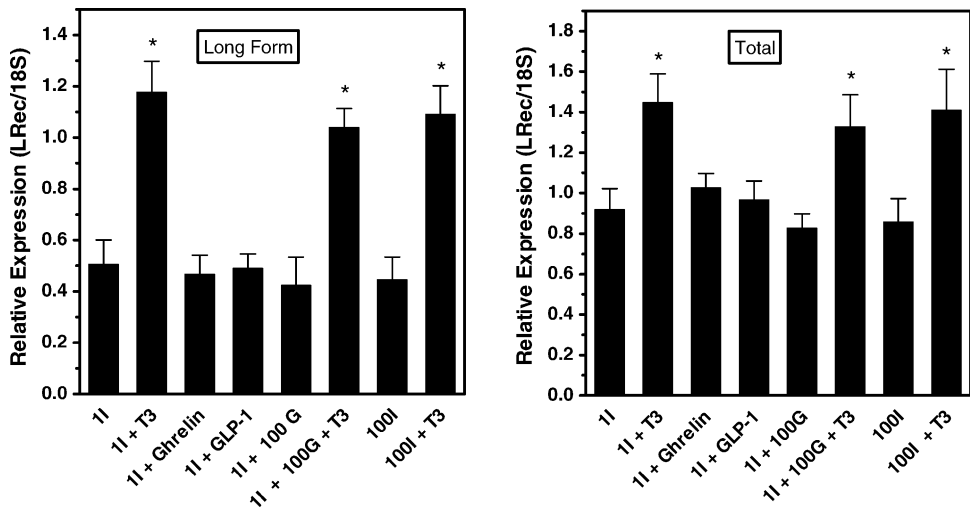


Fig. 2. Influence of hormones on leptin receptor expression in porcine hepatocytes. Hepatocyte monolayers were maintained under basal conditions as described in Section 2. For the final 24 h of incubation the following hormone amendments were added: 1I or 100I (1 or 100 ng/ml bovine insulin); T3 (100 nM triiodothyronine); ghrelin (100 ng/ml synthetic human ghrelin); GLP-1 (100 ng/ml synthetic human GLP-1) or 100 G (100 ng/ml porcine glucagon). Data represent the means and S.E.M. for triplicate cultures from five animals. Means with a “\*” symbol were different from basal cultures (1 ng/ml insulin) as determined by GLM ANOVA and Student’s multiple comparison test ( $P < 0.05$ ).

sion (1.6-fold,  $P < 0.001$ ), while insulin, glucagon, ghrelin and GLP-1 had no influence on total leptin receptor expression. Various levels of T3 ( $10^{-9}$  to  $10^{-6}$  M) were evaluated for leptin receptor induction in cultures of porcine hepatocytes (Fig. 3). While T3 levels greater than 1 nM were found to stimulate all forms of the leptin receptor, 100 nM T3 was chosen as the optimal concentration for subsequent experiments.

The potential influence of short-term leptin exposure on glucagon-induced cAMP production was evaluated in porcine hepatocytes maintained under serum-free conditions. Marked increases in cellular levels of cAMP were obtained when glucagon was added at either 5 or 10 ng/ml for 20 min (Fig. 4). Leptin addition had no influence on cellular levels of cAMP. Further, leptin addition to cultures, where levels of leptin receptor(s) expression were enhanced by exposure to T3 for the previous 24 h, was not associated with alterations in cAMP production. When cellular levels of cAMP were determined at 90 min post-glucagon addition, similar results were obtained (viz., no leptin influence on cAMP) although the overall cellular concentration of cAMP was markedly reduced by that time (data not shown).

The potential influence of leptin on glycogen turnover was evaluated in porcine hepatocyte cultures maintained with 100 ng/ml insulin to elevate the intracellular glycogen concentration (Fig. 5). Addition of glucagon (10 ng/ml) was associated with a loss of approximately 43% of the glycogen in a 3-h period ( $P < 0.001$ ). Simultaneous addition of leptin had no influence on glycogen turnover and there was no interaction between leptin and glucagon addition ( $P < 0.85$ ). In cultures that were pre-incubated with T3 for 24 h (to

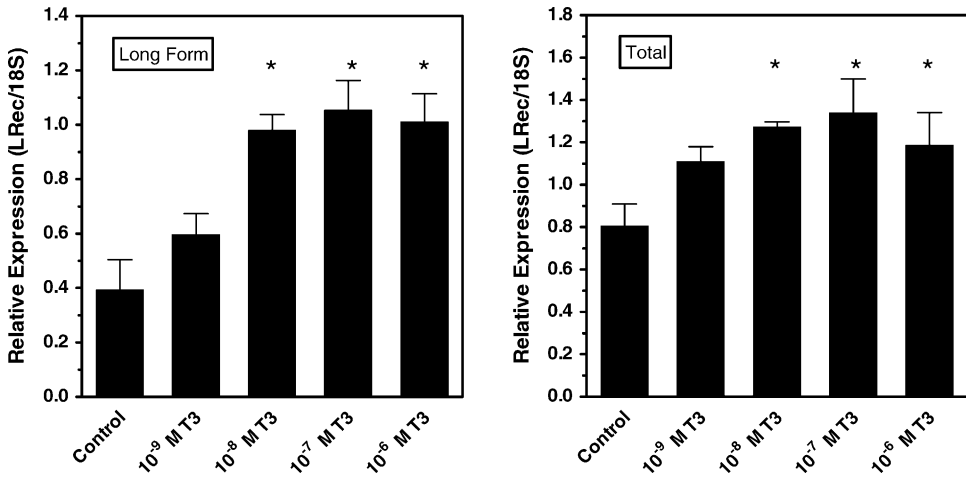


Fig. 3. Influence of triiodothyronine (T3) concentration on leptin receptor expression in porcine hepatocytes. Hepatocyte monolayers were maintained in the presence of 1 ng/ml insulin and various concentrations of T3 for the final 24 h as described in Section 2. Data represent the means and S.E.M. for triplicate cultures from three animals. Means with a “\*” symbol were different from control cultures (basal conditions) as determined by GLM ANOVA and Student’s multiple comparison test ( $P < 0.05$ ).

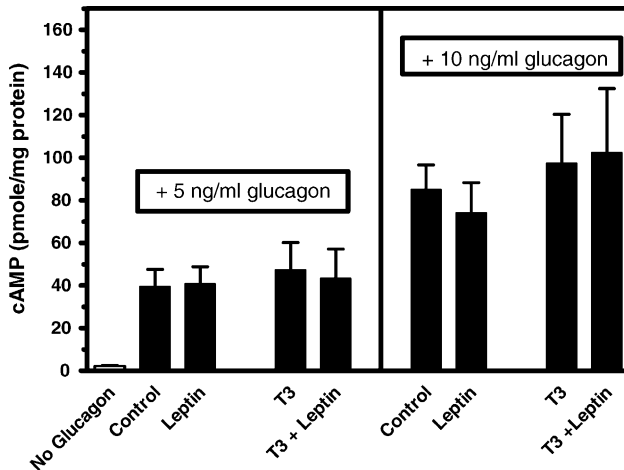


Fig. 4. Influence of glucagon, triiodothyronine (T3) and leptin on cAMP production in porcine hepatocytes. Hepatocyte monolayers were maintained in the presence of 100 ng/ml insulin for the final 24 h of culture either alone (Control) or in the presence of T3 (100 nM), leptin (100 ng/ml) or leptin and T3. Monolayers were washed with warm HEPES-saline and fresh medium containing glucagon at 5 or 10 ng/ml (and the indicated hormone treatment) was added for 20 min at 37 °C. Cells were washed with cold HEPES-saline and were killed by addition of 0.1N HCl and frozen immediately. Data represent the means and S.E.M. for duplicate cultures prepared from three to five animals. Although there was a significant difference between addition of 5 vs. 10 ng/ml glucagon ( $P < 0.001$ ), neither T3 nor leptin (nor both) had any influence on cAMP production as determined by GLM ANOVA. Further, there were no significant treatment interactions when the data were analyzed in a  $2 \times 2 \times 2$  treatment array (leptin  $\times$  T3  $\times$  glucagon) by GLM ANOVA.



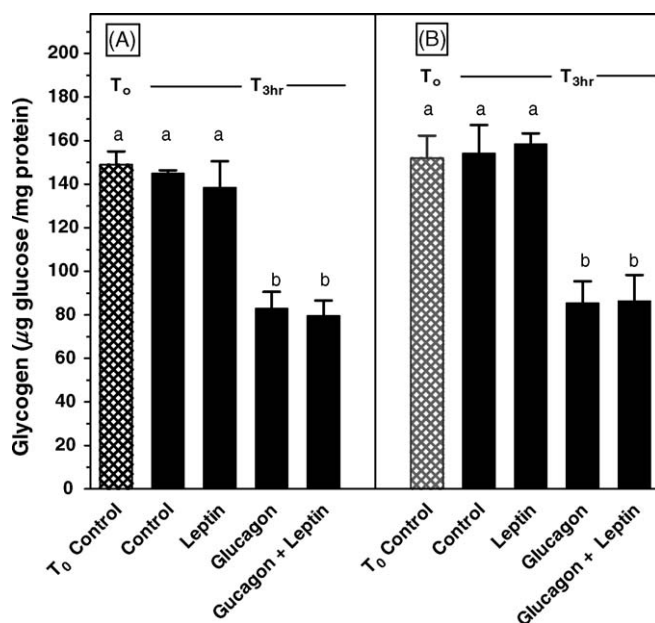


Fig. 5. Influence of leptin and triiodothyronine (T3) on glucagon-induced glycogen turnover in porcine hepatocytes. Hepatocyte monolayers were maintained in the presence of 100 ng/ml insulin (Panel A) or 100 ng/ml insulin and T3 (100 nM) for the final day of culture (Panel B). Following the 24 h pre-incubation period, monolayers were either harvested (T<sub>0</sub> Control) or given fresh medium containing 100 ng/ml insulin, insulin with 100 ng/ml leptin, 10 ng/ml glucagon or glucagon plus leptin (Panel A) or 100 ng/ml insulin and 100 nM T3, with or without 10 ng/ml glucagon and leptin (Panel B). After 3 h of incubation, cells were harvested and glycogen was analyzed as described in Section 2. Data represent the means and S.E.M. for duplicate cultures prepared from three animals. Data were analyzed by GLM ANOVA and the means were separated by Tukey–Kramer Multiple Comparison Test. Means, within each panel with different letters (a,b) differ,  $P < 0.05$ .

elevate leptin receptor expression), leptin addition had no influence on glucagon-induced glycogen degradation.

#### 4. Discussion

In this study we used primary hepatocyte monolayers obtained from pigs in the 50 to 60 kg weight range which were maintained for 48 h in serum-free culture (72 h total) to explore the relationships between the hormone environment and leptin action. Utilizing a highly sensitive quantitative RT-PCR technique we established the presence of the long form of the leptin receptor as well other shorter form(s) in cells from monolayer cultures. The mammalian leptin receptor is a single gene with multiple splice variants that yield a full length transcript (long form) and several shorter isoforms [5]. The porcine leptin receptor sequence has high sequence homology to other mammalian species, and particularly to human [2,7]. Primers used for the determination of leptin receptor transcripts by RT-PCR were derived from gene sequences corresponding to the extra-cellular and intracellular domains



of the porcine protein [2]. Specifically, total leptin receptor transcripts were determined using primers designed from sequence corresponding to a portion of the extra cellular domain region common to all receptor variants, whereas long form transcripts were detected using primers designed from sequence coding a portion of the intracellular domain found only in this variant form. The expression of the long form of the leptin receptor has been previously reported in whole fetal and adult porcine liver [2,7] and in short-term cultures of suckling pig hepatocytes [15]. In addition, the leptin receptor has been identified in many cell types and peripheral pig tissues [2,7]. While the presence of the leptin receptor in porcine liver has been well established, what is lacking is a clear understanding of the function of the leptin receptor in hepatic cells.

We have previously demonstrated that pig hepatocytes are highly sensitive to hormone manipulation *in vitro* but could not establish any influence of leptin on cellular energetics, *viz.*, ketogenesis, redox state, and lipid or glycogen metabolism [8]. In addition, Raman et al. [15] also determined that leptin had no direct effect on hepatic metabolism in pig hepatocytes. In the present study, we addressed this question by first determining whether or not the long form (presumably the most active form) of the leptin receptor was being expressed in our system and then identified which regulatory hormones influenced the expression of the gene. Next we attempted to up-regulate the expression of the receptor (with optimal levels of T3) and investigated whether enhanced transcription of the leptin receptor could alter downstream metabolism by leptin. Incubation for 24 h with the polypeptide hormones insulin, glucagon, GLP-1 and ghrelin had no influence on leptin receptor gene expression. In this system, rh-leptin also did not influence leptin receptor expression (two experiments, data not shown). Similarly, Raman et al. [15] determined that glucagon and leptin had no influence on the expression of long form of the leptin receptor in baby pig hepatocyte cultures. However, in chicken hepatoma cells, insulin and leptin down-regulated leptin receptor expression [19].

In contrast to what was observed for the polypeptide hormones, T3 markedly enhanced leptin receptor expression in porcine hepatocytes. This is the first demonstration of T3 regulation of leptin receptor expression, which may be species-specific since T3 had no influence on leptin receptor expression, relative to 18S rRNA expression, in chicken hepatoma cells [19]. While our data does not allow for direct estimation of the relative expression of long form compared to shorter forms of the leptin receptor expression, it would appear that the long form is differentially induced by T3 since the relative maximal induction of the long form was about 50% greater than that observed for the total forms (2.4 versus 1.6-fold). Although data on the influence of T3 and its effects on leptin receptor expression are lacking, there are several studies which have suggested that thyroid status impacts leptin synthesis. For example, T3 treatment of hypothyroid rats has been shown to reduce circulating leptin primarily through a reduction in fat mass [20]. While in humans, circulating leptin was not altered by 7 days treatment with T3, but leptin expression was depressed in fat biopsies incubated with T3 *in vitro* [21].

Next, we altered leptin receptor expression (total and long forms) by incubating hepatocytes with T3 to determine if leptin could indeed regulate two metabolic processes which have been previously shown to be influenced by leptin in rodents. Zhao et al. [22] demonstrated that leptin attenuated cAMP production following glucagon stimulation in cultured rat hepatocytes. In the present study, low levels of glucagon markedly stimulated cAMP

production, however, leptin alone or combination with T3 in leptin receptor-modulated cells was not associated with altered cAMP induction. Likewise, leptin had no influence on short-term glycogen turnover in the presence or absence of T3, with or without glucagon. This is in sharp contrast to rat hepatocytes where leptin apparently enhanced glycogen deposition by inhibiting phosphorylase activity [9] and also attenuated glucagon-induced gluconeogenesis [15].

In this study, we have demonstrated that the leptin receptor gene is expressed in cells obtained from pigs which were maintained *in vitro* for at least 72 h; moreover, the expression is up-regulated by T3. However, we have not been able to define a function for this receptor in the pig liver. Due to the high sequence homology of human and porcine leptin [23] and reported action of rh-leptin on porcine endothelial [24] and pituitary cells [25] it is unlikely that the lack of effect of leptin in this system is related to a deficiency in the cross-species receptor–ligand interaction but rather reflects the biological role of leptin in the metabolism of porcine hepatocytes. Whether the receptors act as ligand scavengers or are present in too low of a concentration to markedly affect energy metabolism remains to be determined. Our preliminary attempts to determine if actual receptors are being elaborated and expressed on the hepatocyte cell surface by western blot and/or immunoprecipitation analysis using commercially available antibodies have thus far, not been successful. Development of an antiserum specific for the porcine leptin receptor is currently underway in our laboratory. Defining the role of the hepatic leptin receptor can only be accomplished once the protein has been identified and its synthesis and regulation confirmed at the protein level. However, what is already clear is the role of the leptin receptor and thus, leptin, in the regulation of energy metabolism in pig liver is likely to be minimal and could indicate that its metabolic effects on liver tissue may be indirect via the CNS.

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